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IMMUNOFLUORESCENT METHODS IN THE DIAGNOSIS OF INFECTIOUS DISEASES*

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Fluorescent antibody (FA) methods (synonymous with immunofluorescence [IF]) were first developed and used by Coons and associates (1942, 1950a, 1950b) to demonstrate soluble bacterial antigens, viruses and rickettsiae in tissues. However, their application to the field of laboratory diagnosis was pointed out for the first time by Godman [*sic;* should read Goldman] (1953, 1954), who used FA methods in an attempt to differentiate *Entamoeba histolytica* from *Entamoeba coli*. Since then, extensive studies have been made pertaining to the development of FA methods and their role as a serological instrument in the diagnosis of many bacterial, viral, mycotic and parasitic infections of man (Cherry, Goldman, Carski and Moody, 1960). Their potential value lies in the relative *rapidity* with which a diagnosis may be made, in their greater sensitivity compared with established procedures of examination and in their *specificity in detecting the etiological agent in contaminated material.*

In spite of the fact that the principles that govern FA reactions are simple in theory, there are optical and technical complexities associated with the present procedures. The auto-fluorescence of microorganisms and tissues, as well as nonspecific staining, can contribute to raising many problems. These factors require a clear understanding of the principles, methods and limitations of immunofluorescence, before it is possible to perform the test satisfactorily and to make a correct interpretation of the results.

Although none of the FA methods devised at present has replaced the conventional, established methods of laboratory diagnosis, the results of an extensive evaluation of some of the techniques by public health

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laboratories indicate their potentially broad applicability to the field of microbiological diagnosis. Thus the importance of the technologist is evident, both in evaluation and in possible resultant routine applications and the need for a broad knowledge of the scope of diagnostic IF is also emphasized.

Methods and Principles

The fluorescent antibody methods are utilized in microbiological diagnosis to determine either a) the presence of unknown antigens (usually microorganisms) in tissue sections or smears, or b) the presence of an unknown antibody in the serum of the patient. There are four methods by which one or both objectives can be achieved: 1) Direct; 2) Indirect; 3) Inhibition, and 4) Complement staining. The direct and indirect methods have been used to a greater extent than the last two procedures and ~~they will be~~ discussed with more detail. Since the principles of IF depend on the method used, they ~~will be~~ described ~~under the pertinent sections~~.

Direct Method.--This method, which is relatively simple, is used to detect unknown antigens in tissue sections or smears. It is based on the principle that the reaction between an antigen and its pecific antibody globulin, labelled (conjugated) with a fluorescent stain, can be demonstrated by means of coating the antigen with the fluorescent glow of the antibody, as viewed in the microscope with ultraviolet illumination. (The stain normally used in IF studies is *fluorescein isothiocyanate*, which imparts a greenish-yellow fluorescence under the above-indicated immunological conditions.) The tissue section or the smear to be tested with regard to the presence of antigen is fixed to the slide by using one of the various methods, depending on the nature of the material. The specific antibody globulin labelled with fluorescein is added to the antigen at its optimum dilution* and it is allowed to react from 10 to 60 minutes at room temperature or at 35°-37°, depending on the system used. Then the preparation is washed to eliminate the labelled antibody *not attached to the antigen.* The smears or tissue sections are dried with absorbent paper or by draining and the preparation is mounted on a drop of buffered glycerin. When a specific antigen-antibody reaction takes place, washing does not eliminate the attached antibody and the antigen coated with antibody appears under the microscope showing a fluorescent glow. On the other hand, a lack of fluorescence indicates the absence of the specific antigen for the corresponding labelled antibody added to the slide which probably was eliminated by washing.

Adequate controls should be included in each test being made, for the purpose of verifying that an observed positive result represents a specific antigen-antibody reaction. The following list gives the controls included totally or partially in the test systems under study at

***Each lot should be titered against a known positive antibody. The highest dilution causing the most brilliant fluorescence (4+) is the one considered to be optimum.**

present and it also includes the reactions that should be obtained, in order to confirm the specificity:

1. Unknown antigen in a tissue section or smear + labelled "normal" globulin* = lack of fluorescence.

2. Unknown antigen in a tissue section or smear + labelled specific antibody diluted to the optimum degree with a suspension of homologous antigen = lack of fluorescence (inhibition).

3. Unknown antigen in a tissue section or smear + *unlabelled* specific antibody + labelled specific antibody = lack of fluorescence (inhibition).

4. Known specific antigen in a tissue section or smear + labelled specific antibody = fluorescence.

5. Uninfected tissue section or smear + labelled specific antibody = lack of fluorescence.

6. Tissue section or smear with unrelated antigens + labelled specific antibody for testing an antigen system = lack of fluorescence.

The direct method as a means for detecting antigens (microorganisms has been extensively evaluated with regard to a certain number of testing systems. Among them are: 1) Detection of *Neisseria gonorrhoeae* in females (Deacon, Peacock, Freeman and Harris, 1959); 2) Identification of *Streptococcus pyogenes* Group A in pharyngeal smears (Moody, 1959); 3) Detection of the pathogenic serotypes of *Escherichia coli* in the feces of nursing infants with infantile diarrhea (Whitaker, Page, Stulberg and Zuelzer, 1958), and 4) Diagnosis of rabies (Goldwaser [*sic;* should read Goldwasser] and Kissling, 1958). Labelled antibody globulins against each one of these as well as for other microorganisms can be acquired commercially from various sources (Cherry, Goldman, Carski and Moody, 1960).

Indirect Method.--This method can be used either for detecting an unknown antigen in tissue sections or smears or an unknown antibo in a patient's serum. It is based on the principle that an antigen-antibody reaction would become visible by adding to the complex a labelled anti-antibody globulin directed against the antibody globulin of the species used in the test. Fluorescence of the coated antigen, observed microscopically with ultraviolet illumination would result from the antigen + antibody globulin + labelled anti-antibody complex. The indirect method has the advantage of using one single labelled anti-antibody globulin (sometimes referred to as anti-globulin) to detect the specific antigen-antibody reactions that have occurred within a certain species. For example, antihuman globulin obtained from a goat can be used to discover any human antigen-antibody complex. Nevertheless, if the antibody to be tested (or prepared for its use) in the initial reaction was obtained from

*Obtained by fractioning the normal, labelled serum of the globulins.

rabbits, then the labelled anti-globulin in the secondary reaction would have to be prepared against rabbit globulin.

In detecting an unknown antigen, the tissue section or the smear are fixed on the slide as indicated above for the direct method. The unlabelled antiserum specific for the antigen to be tested is added to the antigen and allowed to react for 10-60 minutes at room temperature or at 37°, according to the system. Then the preparation is washed to eliminate the unlabelled antibody *not attached* to the antigen and it is dried with absorbent paper. When human antiserum is used in the initial system, the labelled antiglobulin homologous to the antibody globulin of the animal species (human antiglobulin from a goat) is added to the preparation in its optimum dilution*. Then, procedures of incubation, washing and drying are carried out in the manner described and the smear is mounted on a drop of buffered saline-glycerin solution. When a specific reaction occurs between the antigen and the antibody, washing probably will not eliminate the antibody globulin that is attached and the labelled antiglobulin of the same species will probably be attached in the original complex. Washing cannot remove the attached labelled antiglobulin and the coated antigen appears with a fluorescent glow. On the other hand, a lack of appreciable fluorescence indicates the absence from the preparation of the specific antigen for the corresponding antibody that is unlabelled and, consequently, its elimination by washing. In addition, the labelled antiglobulin is also eliminated due to a lack of attached specific antibody.

In the detection of an unknown antibody in the patient's serum, the procedure is essentially the same, except for the use of a known antigen and the patient's serum in the primary reaction. Titrations could be made by using dilutions of the patient's serum and by recording as the final point the highest dilution that causes a 3+ or 4+ specific fluorescence of the antigen.

In order to verify the specificity of the indirect test, the following are the controls generally used, all or in part, and their anticipated reactions:

1. Known antigen + unlabelled specific antiserums with a variable reactivity + labelled antiglobulin of the species from which the specific antiserum was obtained = fluorescence (whose degrees fluctuate from 4+ to a lack of reactivity).

2. Known antigen or unknown antigen + normal unlabelled serum + labelled antiglobulin from the species with which the specific antiserum was obtained = lack of fluorescence.

***This is determined by titrating each lot in the presence of the known antigen and of the specific antibody. The highest dilution causing the most brilliant fluorescence (4+) is the one considered to be optimum.**

3. Known antigen or unknown antigen + labelled antiglobulin of the same species with which the specific antiserum was obtained = lack of fluorescence.

The most notable application of the indirect method to microbiological diagnosis is in the detection of the fluorescent treponemal antibody in the serum of syphilitic patients, using dead virulent *Treponema pallidum* as antigen (Deacon, Falcone and Harris, 1957). Although it is still in the process of evaluation, the test appears promising as a possible supplement to the battery of serological tests presently in use. A detailed description of this method can be found in the *Manual of Serologic Tests for Syphilis* (1959). The *Treponema pallidum* antigen and undiluted labelled antiglobulins can be acquired commercially (Cherry, Goldman, Carski and Moody, 1960).

Inhibition Method.--This method is most frequently used as a contrast for the specificity of a direct fluorescence procedure. It has also been applied to the detection of *Toxoplasma gondii* antibodies in serum (Goldman, 1957). The test is based on the principle that when the antigen is treated with the unlabelled specific antibody, it becomes saturated. Subsequent exposure of the complex to the *labelled* antibody is probably expressed in a lack of reaction, due to the blocking effect and the antigen *probably will not have fluorescence.* Treatment of the antigen with normal unlabelled serum and subsequent exposure to the labelled specific antibody will probably produce brilliant fluorescence of the antigen. When this procedure is applied for diagnostic purposes, the optimum concentrations of both antibodies, the labelled and the unlabelled, should be determined. In such cases, known positive and negative controls should be used.

Complement Staining Method.--This method is similar to the indirect method, except that the labelled antiglobulin is directed against the species (guinea pig) that supplies the complement. It can be used to detect an unknown antigen in tissue sections or smears or an unknown antibody in the serum of the patient. The test is based on the principle that the antigen is sensitized by the specific antibody and the guinea pig complement combines with the complex. The addition of labelled antiguinea pig globulin determines its fixation to the complex with fluorescences of the antigen.

Each individual specimen to be used as a source of complement should be tested beforehand, for the purpose of avoiding the use of guinea pig serum that causes unspecific fluorescence. Furthermore, in addition to the controls applicable to the indirect method, the following should be included:

1. Known or unknown antigen + unlabelled normal serum + unlabelled guinea pig complement + labelled guinea pig antiglobulin = lack of fluorescence.

2. Known or unknown antigen + unlabelled guinea pig complement + labelled guinea pig antiglobulin = lack of fluorescence.

3. Known antigen + unlabelled specific antiserum + unlabelled guinea pig complement + labelled guinea pig antiglobulin = fluorescence.

4. Known or unknown antigen + guinea pig antiglobulin = lack of fluorescence.

The complement staining method was applied by Goldwasser and Shepard (1958) to the detection of rickettsial antibodies in human serum.

Preparation of Reagents

Antiserum Fractioning and Globulin Labelling

Globulins are normally precipitated from the antiserum by means of the method that use semi-saturation with ammonium sulfate. Then they are labelled with fluorescein isothiocyanate described by Riggs and associates (1958), in accordance with the method recommended by Marshall, Eveland and Smith (1958). The globulin thus prepared can be purified by means of chromatography in column, using DEAE, by means of which the fluorescence of the components of normal tissues, caused by substances inherent in most of the conjugates is reduced considerably. This non-specific fluorescence is commonly associated with the staining of antigens in tissue sections. The adsorption of conjugates with powdered tissue dried with acetone or lyophilized (available commercially) is used, in addition to chromatography in DEAE column, to reduce the background non-specific fluorescence. In some cases, both methods are required.

Fluorescein isothiocyanate is the label that is used most extensively, due to the ease and convenience with which the greenish-yellow fluorescence that it imparts to antigens coated with antibody can be observed. Besides, the auto-fluorescence of tissues and microorganisms is rarely observed within this spectrum.

When it is desirable to locate more than one antigen in a preparation, each specific antibody globulin directed against a particular unknown antigen could be labelled with a fluorescent dye that is different in color contrast and a combined labelled globulin could be added to the tissue section or smear. Dyes with a red fluorescence, like rhodamine B isothiocyanate and rhodamine B 200 lysamine, can be used for these purposes. In addition, some of these red dyes can be used for background contrast staining. The use of these dyes with fluorescein isothiocyanate makes possible a better visualization of the greenish-yellow fluorescence that indicates a specific antigen-antibody reaction.

A variable reduction in the original antibody titer resulting from the conjugation of the globulin indicates a need for testing the staining and optimum dilution characteristics of each lot, whether acquired commercially or prepared in the laboratory. Satisfactorily labelled globulins probably retain their potency and characteristics for long periods of time when they are lyophilized and kept at -20° or are stored at -20° undiluted

in sufficient quantities for use without refrigeration.

Labelled Globulin from Anti-Human Goat Serum (for the Indirect Test)

Proom's (1943) method is utilized to immunize goats by means of a series of intramuscular injections of normal human serum precipitated by alum. Then the goats are bled from the jugular vein. The globulin is obtained from the serum and is labelled as described above.

Microscopy

Most researchers have found the use of dark field illumination more satisfactory than bright field illumination for work with a fluorescent antibody. The use of the condenser in a dark field makes better visualization possible, avoiding the entrance of excessive ultraviolet illumination in the microscopic field which would thus mask the weak fluorescent color of the material. Contrast of objects against the completely dark background is favored in this way.

Although the selection of a monocular or binocular eyepiece would depend on the particular method followed, most researchers use the monocular tube. The use of a binocular eyepiece would result in a forty to fifty percent sacrifice of the fluorescent glow, thus interferring considerably with the sensitivity of the system.

The most appropriate choice of excitation (primary) and barrier (secondary) filters is extremely important for eliminating or reducing the problem of auto-fluorescence inherent in certain microorganisms and tissues, without affecting the fluorescence due to the specific antigen-antibody reaction. For example, the use of a Corning 5840 excitation filer and a Wratten 2A gelatine barrier filter (ocular) in the fluorescent antibody test for rabies is appropriate for obtaining the sharpest contrast between the greenish-yellow fluorescence, typical of the specific antigen-antibody reaction, and the bluish-gray or whitish-blue auto-fluorescence of the cells. The use of colored barrier filters would cause the auto-fluorescent cells to appear also with a greenish-yellow tint, with a consequent interference with an accurate interpretation of the results.

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